Isolation and Identification of Seed-Borne Fungi of Maize Grains from Cultivated in Phitsanulok Province, Thailand

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Abstract

Maize is an important agricultural product in Thailand. Most of the extracts from maize serve as the main element for animal feed while others are used in several recipes. Prior studies found that maize is contaminated by fungi including *Fusarium*, *Aspergillus* and *Penicillium* which produce toxins affect human and animal. The main objective of this study was to isolate and identify seed borne fungi in maize grain cultivated in different districts of Phitsanulok province. The samples were collected from three sample sites in each districts in the Phitsanulok province namely, Mueang, Wangthong, Nakhonthai, Noenmaprang, Bangkrathum, Bangrakam, and Phromphiram districts. The maize grains were disinfected with 5% sodium hypochlorite solution. PDA plating method was conducted for the isolation of the fungi and cultural, a microscopic study was adopted for the identification. The seeds were highly contaminated with five fungal species (Fusarium, Penicillium, Aspergillus, Rhizomucor, and Mucor) were detected in maize grains. *Aspergillus* spp. (28.07%) was the most dominant in maize grain from Noenmaprang and followed by those from Nakhonthai (17.65%), Mueang (15.12%), Wangthong (12.38%), Phromphiram (6.14%), and Bangkrathum (4.17%), respectively. However, *Aspergillus* spp. were not detected in in maize grain from Bangrakam. While, Fusarium spp. were highly presented in maize grain from Wangthong (9.52%) and followed by those from Bangkrathum (7.50%), Nakhonthai (4.2%), Noenmaprang (1.75%), and Bangrakam (1.67%), respectively. In contrast, *Fusarium* spp.were not detected in maize grain from Mueang and Phromphiram. In addition, the *Rhizomucor* spp. was the most common pathogen in the Bangrakam district. The *Mucor* spp. was more present in the Nakhonthai district.

Keywords: Isolation, Identifications, Seed-borne fungi, Maize, Phitsanulok

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Introduction

Maize is an important cereal grown worldwide and is used as feed for livestock such as poultry and pigs (Islam *et al*., 2015, Kanengoni *et al*., 2015). It has a high content of nutrients and consists of about 50-70% of poultry feeds. (Krnjaja *et al*., 2017). Besides, it is the most important source of energy in pig feeds (Kanengoni *et al*., 2015). Unfortunately, it is frequently infected by a variety of toxigenic fungi (Marín *et al*., 2012). Mycotoxin contamination of maize grain during and post-harvest is facilitated by environmental and climatic factors (Krnjaja *et al*., 2017).

Factors that promote the growth and spread of mycotoxigenic fungi in maize grain pre and post-harvest include moisture content and temperature (Krnjaja *et al*., 2017). Prior investigations revealed that *Fusarium*, *Aspergillus* and *Penicillium* are some of the toxigenic species that affect maize grain (El-Shanshoury *et al*., 2014, Camardo Leggieri *et al*., 2015). The toxins they produce include fumonisins, zearalenone, aflatoxins, and ochratoxin (Zain, 2011). These contaminants could lead to nutrient loss and can affect animal production, growth, milk production, performance, and fertility. Kehinde *et al*. (2014) determined aflatoxin in five samples of animal feed in Abeokuta using enzyme-linked immunosorbent assay (ELISA). It has been found that maize is the main ingredient in all the contaminated feed. The mean aflatoxin level was observed to be highest in poultry feed from Lafenwa (93.1 μg/kg) and lowest in that from Idi-Aba with mean value of 13.5 μg/kg.

In poultry, maize comprises a major part of feed ingredient. Fumonisin contamination is associated with reduced performance in poultry production and may be characterized by diarrhea and decrease in body weight, and egg production as previously reported in India (Weibking *et al*., 1993). Fumonisin contamination was investigated in maize and poultry feeds in Haryana, India. Fumonisin B1 contamination in the maize samples ranged from 0.1–87.0 ppm. However, the concentrations in poultry feed samples range from 0.02–28.0 ppm (Jindal *et al*., 1999). Moreover, several studies have reported the adverse effects of aflatoxins on birds including a reduction in performance, pathologic alterations in the liver and kidneys as well as the immune system of birds (Abidin *et al*., 2011). According to a previous study, a dose of 0.25 ppm of aflatoxins in turkey and ducklings impairs growth, and a dose of 1.5 ppm in broilers and 4 ppm in Japanese quail negatively affect growth (Algabr *et al*., 2018). In pig, the damage caused by zearalenone (ZEA) can be high because its metabolites have oestrogenic and anabolic activities which may cause hyperestrogenism and affect the animal reproductive system. Not very much has been done to compare pathogenic fungi in Thailand. Therefore, this study aimed to isolate and identify seed-borne fungi in maize grain cultivated in different districts of Phitsanulok province.

Materials and methods

Sample collection

Maize grain samples were collected from the main production areas located in the Phitsanulok province. These areas included Mueang, Wangthong, Bangrakam, Bangkrathum, Phromphiram, Noenmaprang, and Nakhonthai districts. Each district had three sample locations. Seed-borne fungi were isolated from at least 100 grains of maize samples from each district.

Fungal screening from maize grains

Maize samples were randomly selected from three locations in each of the 7 maize growing districts in Phitsanulok province as follows: Mueang Phitsanulok (n = 119), Wangthong (n = 105), Bangrakam (n = 120), Bangkrathum (n=120), Phromphiram (n = 114), Noenmaprang (n = 114), and Nakhonthai (n = 119). The maize grains were soaked in 5% sodium hypochlorite solution (NaOCl) for about 15 minutes. They were then immersed in sterile distilled water (SDW) for 1 minute and were rinsed with SDW for another 1 minute. Eight to twelve samples were then dried using sterilized filter papers (Whatman No 1) and were placed on potato dextrose agar (PDA) plates. The PDA plates were incubated at 28 °c for 7 days. The seed-borne fungi that developed after incubation of each grain were counted directly. Moreover, those of different species were sub-cultured on PDA at 28 °c for 5 days. The pure colonies were isolated for further identification using microscopic observation/Lactophenol cotton blue staining technique (Figure 1).

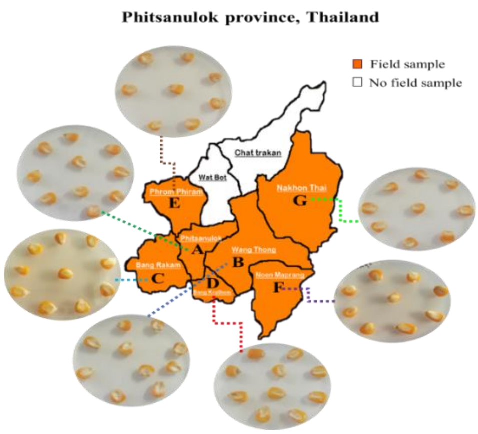

Figure 1 The collection of maize grains were sampling in each district located in Phitsanulok province. A) Mueang, B) Wangthong, C) Bangrakam, D) Bangkrathum, E) Phromphiram, F) Noenmaprang, and G) Nakhonthai districts. *Morphology of seed-borne fungi prepared using the Lactophenol cotton blue staining technique*

A drop of lactophenol blue solution was placed on a glass slide. Pure culture of the toxigenic seed-borne fungi was removed using a sterile inoculation loop and then transferred to the slide. The culture was teased out very gently using a second needle. A coverslip was gently placed on the slide to avoid air bubbles. The seed-borne fungi were identified on the basis of sporemorphology and mycelium characteristics using stereoscopic-binocular microscope. A list of morphological characters of taxonomic importance such as type of spore, septation, color and character of the mycelium were compiled for each fungus.

Statistical analysis

The percentage of seed-borne fungi in maize analyzed by using SPSS statistics 19 software. Analysis of variance (ANOVA) was performed using general linear model (GLM) procedure.

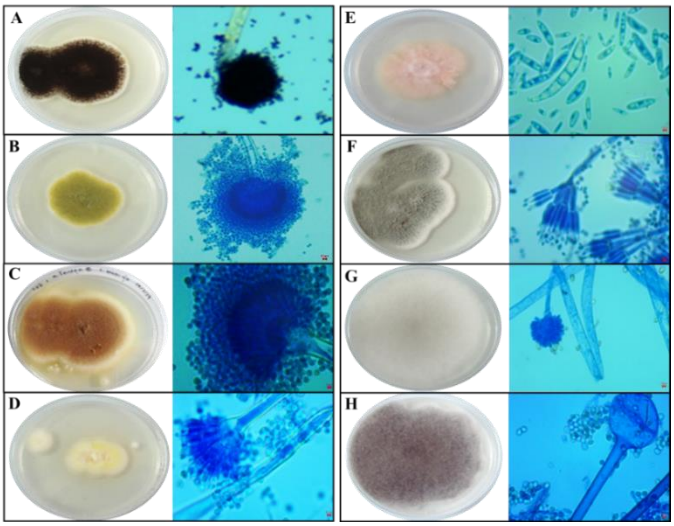

Figure 2 Photographs of the colonies and morphological characteristic of the isolates. The seed-borne fungi incidence in maize grains collected from the seven districts of Phitsanulok province. (A) The *Aspergillus* spp. that haddark-brown to black conidia, (B) *Aspergillus* spp. that the colonies were yellow-green, (C) *Aspergillus* spp. that the colonies were suede-like and cinnamon-buff to sand-brown with a yellow to deep dirty brown reverse. (D) *Aspergillus* spp. that the colony was yellow and had white mycelia at edges, (E) The *Fusarium* spp. that presented multicellular distinctive sickle-shaped macro conidia, (F) The *Penicilium* spp. that consisted of conidium-bearing hyphae or conidiophores, (G) The Mucor spp. that appeared cream white and almost covered the whole surface (H) The Rhizomucor spp., that had a cotton-candy like appearance and well-developed rhizoids and spherical sporangia

Results

Isolation of seed-borne fungi from maize grains by morphological characteristics

Seed-borne fungi incidence in maize across the seven districts was reported. Our investigation showed that all the maize samples were contaminated with at least one strain of fungi. However, co-contamination was common in most of the maize grains. Five species of the toxigenic seed-borne fungi namely *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Mucor* spp., and *Rhizomucor* spp. were isolated from the maize grains (Figure 2). The pathogenic organisms isolated from the maize grains and their morphological characteristics are shown in Table 1. They included 1) the *Aspergillus* spp. that haddark-brown to black conidia (Figure 2A), the colonies were yellow-green and had white mycelia at the edges; sporulation rings; and the slightly rough conidia. (Figure 2B), Besides, the colonies were suede-like and cinnamon-buff to sand-brown with a yellow to deep dirty brown reverse. Conidial heads were found to be compact, columnar and biseriate (Figure 2C), and the colony was yellow and had white mycelia at edges. Conidial heads were compact, columnar and biseriate whereas the Conidia were globose to ellipsoidal (Figure 2D). 2) The *Fusarium* spp. *that* presented multicellular distinctive sickle-shaped macro conidia (Figure 2E). 3) The *Penicilium* spp., that consisted of conidium-bearing hyphae or conidiophores (Figure 2F). 4) The *Mucor* spp. that appeared cream white and almost covered the whole surface (Figure 2 G). 5) The *Rhizomucor* spp. that had a cotton-candy like appearance and well-developed rhizoids and spherical sporangia (Figure 2 H).

The seed-borne fungi incidence in maize grains harvested in Phitsanulok province

Maize samples collected from three locations in each of the 7 maize growing districts were as follows: Mueang (n = 119), Wangthong (n =105), Bangrakam (n = 120), Bangkrathum (n = 120), Phromphiram (n = 114), Noenmaprang (n = 114), and Nakhonthai (n = 119). The highest incidence of seed-borne fungi was in Nakhonthai (56.30%) followed by Noenmaprang (48.24%), Wangthong (42.85%), Bangrakam (30.84%), Bangkrathum (24.17%), Mueang (15.96%), and Phromphiram (7.89%), respectively (Table 1, Figure 3).*Aspergillus* spp. (28.07%) was the most dominant in maize grain from Noenmaprang and followed by those from Nakhonthai (17.65%), Mueang (15.12%), Wangthong (12.38%), Phromphiram (6.14%), and Bangkrathum (4.17%), respectively (Table 1). However, *Aspergillus* spp. was not detected in in maize grain from Bangrakam. While, Fusarium spp.was highly presented in maize grain from Wangthong (9.52%) and followed by those from Bangkrathum (7.50%), Nakhonthai (4.2%), Noenmaprang (1.75%), and Bangrakam (1.67%), respectively (Table 1). However, Fusarium spp. were not detected in maize grain from Mueang and Phromphiram. *Penicillium* spp. have the highest presented in maize grain from Bangkrathum (11.67%) and followed by Wangthong (11.43%), Nakhonthai (4.20%), Noenmaprang (1.75%), Mueang (0.84%), respectively. Bangrakam was not detected in maize grain (Table 1).

The *Aspergillus* spp. was the most dominant fungi in Noenmaprang (28.07%), Nakhonthai (17.65%), Mueang (15.12%), Wangthong (12.38%), and Phromphiram (6.14%). However, the Aspergillus spp. was not present in maize collected from the Bangrakam district (Figure 5). The *Rhizomucor* spp. was the dominant fungi in maize samples from Bangrakam district (29.17%). Moreover, it was also found in maize samples collected from other districts such as the Noenmaprang (3.51%), Nakhonthai (1.68%), and Bangkrathum (0.83%) districts. There was the massive presence of *Mucor* spp. in samples collected from the Nakhonthai (28.57%), Noenmaprang (13.16%), and Wangthong (9.52%) districts, respectively. The *fusarium* spp. was present in samples collected from maize growing districts except the Mueang and Phromphiram districts. Moreover, there was the presence of *Penicillium* spp. in all except the Bangrakam district (Figure 4).

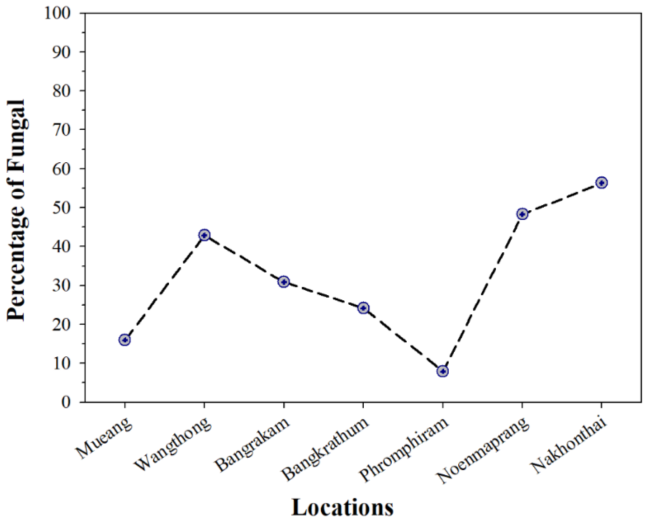

Figure 3 Percentage of seed-borne fungi in maize grains according to the districts

Discussion

Maize is an important food source for humans and serves as the most important raw material for animal feed. Contamination of raw feed materials by mould and mycotoxin is common. This leads to nutrient loss and would have a detrimental effect on animal production. Prior findings revealed that drought, humidity, temperature, insect, infestation, and rough handling are some of the factors that facilitate mycotoxin contamination of agricultural products (Rosemary *et al*., 2013).

Table 1 The seed-borne fungi incidence in maize grains collected from the seven districts in Phitsanulok province

Sample location	No. of maize	Percentage of seed-borne fungi in maize	Fungal isolates
Mueang	119	0(0/119)[c]	*Fusarium* spp.
		15.12(18/119)[bc]	*Aspergillus* spp.
		0.84(1/119)[c]	*Penicillium* spp.
		0(0/119)[c]	*Mucor* spp.
		0(0/119)[b]	*Rhizomucor* spp.
Wangthong	105	9.52(10/105)[a]	*Fusarium* spp.
		12.38(13/105)[bc]	*Aspergillus* spp.
		11.43(12/105)[ab]	*Penicillium* spp.
		9.52(10/105)[b]	*Mucor* spp.
		0(0/105)[b]	*Rhizomucor* spp.
Bangrakam	120	1.67(2/120)[bc]	*Fusarium* spp.
		0(0/120)[d]	*Aspergillus* spp.
		0(0/120)[c]	*Penicillium* spp.
		0(0/120)[c]	*Mucor* spp.
		29.17(35/120)[a]	*Rhizomucor* spp.
Bangkrathum	120	7.50(9/120)[ab]	*Fusarium* spp.
		4.17(5/120)[cd]	*Aspergillus* spp.
		11.67(14/120)[a]	*Penicillium* spp.
		0(0/120)[c]	*Mucor* spp.
		0.83(1/120)[b]	*Rhizomucor* spp.
Phromphiram	114	0(0/114)[c]	*Fusarium* spp.
		6.14(7/114)[bc]	*Aspergillus* spp.
		1.75(2/114)[c]	*Penicillium* spp.
		0(0/114)[c]	*Mucor* spp.
		0(0/114)[b]	*Rhizomucor* spp.
Noenmaprang	114	1.75(2/114)[bc]	*Fusarium* spp.
		28.07(32/114)[a]	*Aspergillus* spp.
		1.75(2/114)[c]	*Penicillium* spp.
		13.16(15/114)[b]	*Mucor* spp.
		3.51(4/114)[b]	*Rhizomucor* spp.
Nakhonthai	119	4.2(5/119)[bc]	*Fusarium* spp.
		17.65(21/119)[bc]	*Aspergillus* spp.
		4.20(5/119)[bc]	*Penicillium* spp.
		28.57(34/119)[a]	*Mucor* spp.
		1.68(2/119)[b]	*Rhizomucor* spp.

The values following by the same letter within the column or row are not significantly different at 0.05 probability level.

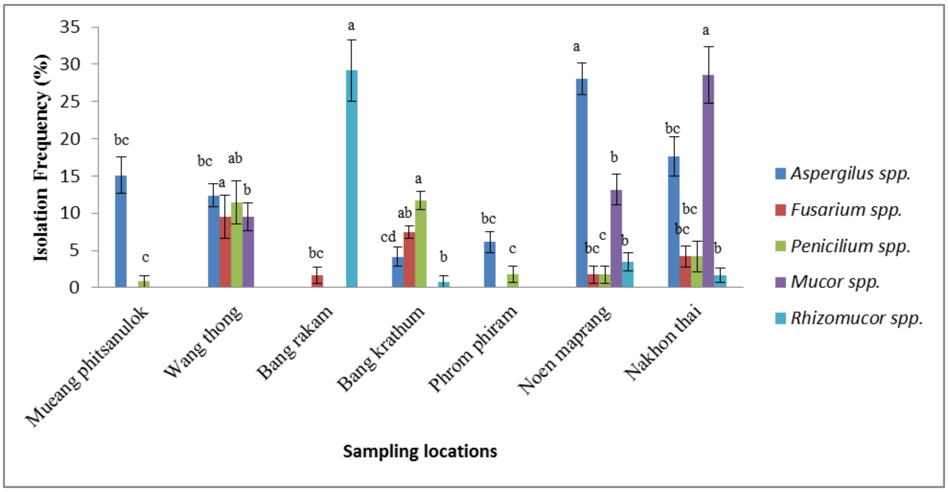

Figure 4 Frequency of occurrence of seed-borne fungi isolated from maize grains collected from the seven districts such as Mueang, Wangthong, Bangrakam, Bangkrathum, Phromphiram, Noenmaprang, and Nakhonthai districts in Phitsanulok province. The values following by the same letter are not significantly different at 0.05 probability level.

In this study, maize samples were collected from three sample locations in each of the 7 maize growing districts in Phitsanulok province (Mueang, Wangthong, Bangrakam, Bangkrathum, Phromphiram, Noenmaprang, and Nakhonthai districts). Seed-borne fungi were identified by their cultural and morphological characteristics as presented in Figure 2. These isolates included *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Mucor* spp., *and Rhizomucor* spp. (Figure 4). Furthermore, the results of this study showed that the highest incidence of the pathogens was in the Nakhonthai district (56.30%) followed by Noenmaprang (48.24%), Wangthong (42.85%), Bangrakam (30.84%), Bangkrathum (24.17%), Mueang (15.96%), and Phromphiram (7.89%) district, respectively (Table 1). Therefore, the different areas in Phitsanulok province affected the percentage of fungal occurrence. In all samples analyzed in Phitsanulok province, the widespread isolate was *Aspergillus* spp. Its dominance could be linked to the incorrectly dried maize (Rosemary *et al*., 2013) and the subtropical to warm temperate zones at attitudes between 26 and 35 °c. This distribution is attributed to several biotic and abiotic interacting factors with the major factor temperature (Klich, 2002, Palencia, 2010). The *Aspergillus* spp. was the most dominant seed-borne fungi in the Noenmaprang district whereas the *Rhizomucor* spp. was the most common pathogen in the Bangrakam district. The *Mucor* spp. was more present in the Nakhonthai district. The *fusarium* spp. was present in all except the Mueang and Phromphiram districts. Also, the *Penicillium* spp. was present in all except the Bangrakam district. Prior works have reported similar isolates in stored maize grains

(Tsedaley and Adugna, 2016, Orsi *et al*., 2000). Aspergillus, Fusarium, and Penicillium are the dominant toxigenic species that affect maize grain. Contamination of maize by toxigenic fungi can occur in the field during harvest and storage (Zorzete *et al*., 2008). Therefore, the quality of maize grain as the most important nutrient in animal nutrition is necessary as a preventive measure to reduce and control contamination of grain with mycotoxigenic fungi

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